Effect of the combinations between pea proteins and soluble fibres on cholesterololaemia and cholesterol metabolism in rats

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Abstract

Many functional foods and dietary supplements have been reported to be beneficial for the management of dyslipidaemia, one of the major risk factors for CVD. Soluble fibres and legume proteins are known to be a safe and practical approach for cholesterol reduction. The present study aimed at investigating the hypocholesterolaemic effect of the combinations of these bioactive vegetable ingredients and their possible effects on the expression of genes regulating cholesterol homeostasis. A total of six groups of twelve rats each were fed, for 28 d, Nath’s hypercholesterolaemic diets, differing in protein and fibre sources, being, respectively, casein and cellulose (control), pea proteins and cellulose (pea), casein and oat fibres (oat), casein and apple pectin (pectin), pea proteins and oat fibres (pea + oat) and pea proteins and apple pectin (pea + pectin). Administration of each vegetable-containing diet was associated with lower total cholesterol concentrations compared with the control. The combinations (pea + oat and pea + pectin) were more efficacious than fibres alone in modulating cholesterololaemia (−53 and −54 %, respectively, at 28 d; P<0.005). In rats fed the diets containing oat fibres or apple pectin, alone or in combination with pea proteins, a lower hepatic cholesterol content (P<0.005) and higher hepatic mRNA concentrations of CYP7A1 and NTCP were found when compared with the control rats (P<0.05). In summary, the dietary combinations of pea proteins and oat fibres or apple pectin are extremely effective in lowering plasma cholesterol concentrations in rats and affect cellular cholesterol homeostasis by up-regulating genes involved in hepatic cholesterol turnover.

Key words: Pea proteins; Soluble fibres; Cholesterol; Rats

Dyslipidaemia is one of the major risk factors for CVD, the leading cause of death in Western populations and a constantly growing cause of morbidity and mortality worldwide(1). Lifestyle changes, one of which is diet, remain the cornerstone for the treatment of dyslipidaemia and the prevention of CVD. Lifestyle changes, in fact, may reduce the risk of premature CHD by 82 %, whereas dietary changes alone may reduce the risk by 60 % (2).

A large number of functional foods and dietary supplements have been reported to be beneficial for the management of dyslipidaemia (3,4). Among those, dietary fibres are recommended as a safe and practical approach for cholesterol reduction. This activity is limited to some soluble fibres, whereas insoluble fibres do not exert this effect (5). Soluble fibres known to lower LDL-cholesterol include pectins from apple and citrus fruits, β-glucan from oats and barley, and fibres from flaxseed and psyllium (6). Based on meta-analyses showing their hypocholesterolaemic activity (7), health claims exist for both oat β-glucan and barley β-glucans for cholesterol reduction (8,9). The mechanisms behind this effect are not fully understood: bile acid binding has been suggested, but there are also other potential means for the reduction (10).

Substitution of animal proteins with vegetable proteins appears to be associated with a lower risk of CHD. Soya protein consumption has been shown to successfully reduce cholesterololaemia in experimental animals, when increased by dietary means (11), as well as in human subjects with cholesterol elevations of genetic or non-genetic origin (12,13). This hypocholesterolaemic effect is not limited to soya, but it appears to be a feature of most legume proteins. Indeed, a strong cholesterol reduction has been shown in animals fed proteins purified from lupin and pea seeds (14,15). A potentially effective strategy for cholesterol reduction through the diet is that of combining various cholesterollowering foods in one diet (16). The present study attempted to evaluate, in a recognised rat model of hypercholesterolaemia, the efficacy of a combination of legume proteins and soluble fibres. Pea proteins were selected, together with β-glucan from oat and pectin from apples. Pea proteins appear to provide an attractive alternative to soya proteins in view of the wide

Abbreviations: ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; CYP7A1, cholesterol 7a-hydroxylase.

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availability, low cost and appropriate taste for the non-oriental population\(^{(19)}\). Vegetable protein and fibres were administered with the diet, alone or in combination, to evaluate a possible additive or synergistic effect. Moreover, mechanisms behind the hypocholesterolaemic effect were explored through real-time quantitative PCR analyses.

**Methods**

**Animals, diets and experimental protocol**

Procedures involving animals and their care were conducted in accordance with the institutional guidelines that are in compliance with national (D.L. no. 116, G.U. Suppl. 40, 18 February 1992, Circolare no. 8, G.U. Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, 12 December 1987; Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, NIH Publication no. 85–23, revised 1996). The experiments were supervised by the Laboratory Animal Welfare Service at our Department.

A total of seventy-two male Sprague–Dawley rats (body weight 200–225 g; Charles River Italia) were housed in a room with controlled lighting (12 h/d), constant temperature and relative humidity. During the first week, they were fed a commercial non-purified diet (Mucedola) and then divided into six groups of twelve rats on the basis of body weight so that the distribution between the groups was similar. The animals were then fed for 28 d ad libitum with Nath’s\(^{(17)}\) hypercholesterolaemic diet (control group) or diets identical to Nath’s diet except for the protein or fibre content. The composition of the experimental diets is shown in Table 1. The protein content in each diet (20 %) was either the total protein isolate from *Pisum sativum* (Pisane\(^{w}\) C9; Provital Industrie SA) or casein as the control protein. Pisane\(^{w}\) Classic AU-201-USP used had a Staudinger index (intrinsic viscosity) of 500 cm\(^3\)/g (Dr Hans-Ulrich Endress, Herbstreith & Fox, personal communication, 2012). Oat fibres were included in the diet as a component of a dietary product, Oatwell\(^{w}\) 28 %, an oat bran rich in β-glucan with a high viscosity and a high molecular weight (CreaNutrition AG). Oatwell\(^{w}\) 28 % contains 28 % β-glucan and 46 % of total fibre. The relative amount of Oatwell\(^{w}\) 28 % in the rat diet was 11·6 %, thus the total fibre content of the diet was 5·3 % and the β-glucan content was 3·25 %. Considering the composition of Oatwell\(^{w}\) 28 %, the diets containing this product were also balanced for minerals, fat and starch content (Table 1). During the feeding period, body weight and food intake were recorded.

**Sample collection**

Fasting blood samples were collected into tubes containing 0·1 % (w/v) EDTA before and after 14 and 28 d of dietary treatments. Plasma was separated by centrifugation at 8000 rpm for 10 min at 4°C and stored at −20°C for lipid analysis. At the end of the dietary treatments (28 d), rats were killed and the liver was excised and immediately snap-frozen in liquid N\(_2\) for subsequent RNA isolation and analysis.

**Plasma and liver lipid measurement**

Plasma total cholesterol, HDL-cholesterol and TAG concentrations were measured with standard enzymatic techniques using a Roche Diagnostics Cobas autoanalyzer. HDL-cholesterol was measured after the precipitation of apoB-containing lipoproteins with polycethylene glycol (20 %, w/v) in 0·2 m-glycine (pH 10). This method has been extensively used for the measurement of HDL-cholesterol concentrations in mice\(^{(20–23)}\) and has been validated in our laboratory for HDL-cholesterol quantification in rats by comparison with results obtained by fast protein liquid chromatography separation of lipoprotein fractions\(^{(28)}\). VLDL + LDL-cholesterol concentrations were calculated as the difference between the total cholesterol and HDL-cholesterol concentrations.

Lipids were extracted from the liver with a mixture of chloroform–methanol\(^{(24)}\); lipid extracts were dried and the lipids dissolved using Triton X-100. Hepatic cholesterol and TAG contents were quantified by enzymatic assays (ABX Diagnostics).

**Table 1.** Composition of the experimental diets

<table>
<thead>
<tr>
<th>Ingredients (g/kg)</th>
<th>Control (Nath’s diet)</th>
<th>Pea</th>
<th>Oat</th>
<th>Pectin</th>
<th>Pea + oat</th>
<th>Pea + pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pea proteins</td>
<td>200</td>
<td></td>
<td></td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Hegsted mineral mix</td>
<td>40</td>
<td>40</td>
<td>35</td>
<td>4-0</td>
<td>3.5</td>
<td>40</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td></td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Sucrose</td>
<td>385</td>
<td>385</td>
<td>385</td>
<td>385</td>
<td>385</td>
<td>385</td>
</tr>
<tr>
<td>Starch</td>
<td>31</td>
<td>31</td>
<td></td>
<td></td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>75</td>
<td>75</td>
<td></td>
<td></td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Pectin</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OatWell(^{w}) 28 %</td>
<td>-</td>
<td></td>
<td>116</td>
<td></td>
<td>116</td>
<td></td>
</tr>
</tbody>
</table>

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**Real-time PCR analysis**

Total RNA was isolated from the rat liver using the NucleoSpin RNA extraction kit (Macherey-Nagel) according to the manufacturer’s instructions. RNA concentration and purity were estimated from optical density at 260 and 280 nm, respectively. Total RNA (1 μg) was reverse transcribed with random hexamer primers and MultiScribe reverse transcriptase (Applied Biosystems) following the manufacturer’s instructions. Complementary DNA were quantified by real-time detection PCR on an Applied Biosystems 7900 sequence detector using SYBR® Green I and specific primers indicated in Table 2. Real-time detection was performed in a volume of 25 μl containing 100 nmol/l of each primer and an iTaq SYBR Green Supermix with ROX 2X as recommended by the manufacturer (Bio-Rad).

**Statistical analysis**

Data are expressed as mean values and standard deviations. Group differences were tested for statistical significance by multivariate ANOVA (repeated measures), followed by Tukey’s post hoc test. *P*<0·05 was considered as statistically significant. Statistical analysis was performed using SYSTAT software (version 13; Systat Software, Inc.).

**Results**

**Effect of the diets on lipid concentrations in plasma and liver**

The vegetable proteins and fibres affected the body weight of the animals differently on the high-cholesterol regimen, when monitored during the 28 d of dietary treatment. The animals fed pea proteins and pea proteins + oat fibres grew significantly more than the control animals (mean weights at 28 d of the diet: 388·92 (SD 23·92) g in controls, 441·83 (SD 26·44) g in pea protein-fed rats and 442·92 (SD 27·78) g in pea protein + oat fibre-fed rats, *P*<0·005). This higher increase in body weight was consequent to a higher food intake (daily food intake: 18.26 (SD 1·45) g/d in controls, 22·29 (SD 3·63) g in pea protein-fed rats and 22·58 (SD 2·82) g in pea protein + oat fibre-fed rats, *P*<0·005).

Each dietary regimen containing vegetable proteins and/or fibres led to lower total cholesterol concentrations when compared with the controls at both 14 and 28 d of the hypercholesterolaemic diet (Fig. 1(a)). The two combinations (pea proteins + oat fibres and pea proteins + apple pectin) proved more effective than fibres alone in modulating cholesterolaemia.

![Image](https://doi.org/10.1017/S0007114513000639)

**Table 2. Sequences of the primers used for real-time PCR analysis**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (from 5' to 3')</th>
<th>Reverse primer (from 5' to 3')</th>
</tr>
</thead>
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<tr>
<td>ABCG5</td>
<td>TGGACTGTCATGACCTGCAAAAT</td>
<td>GAAACAAACCTCTCCGGTAAAG</td>
</tr>
<tr>
<td>ABCG8</td>
<td>GCTCAGTTCAAGTTACGGTG</td>
<td>CTGCCCTGTAACGGTGAG</td>
</tr>
<tr>
<td>BSEP</td>
<td>TCTTGAACCTCTCTTGGCCTTTT</td>
<td>GCCCCAGACTCCTGAGCTCA</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>AGCACCTGGGGGAAAGGATT</td>
<td>AGCCACCTGACTTGGCAGT</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>CACATCTCTGCAACCTTTT</td>
<td>GATCCGGACCGGTGATT</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>CCGACTACATCAACCTGGA</td>
<td>CCACTGGACCTGGTACTCT</td>
</tr>
<tr>
<td>LDL receptor</td>
<td>CAGCTTCTGGTGAACTGGGA</td>
<td>TCTCGGGTGGCGGAGT</td>
</tr>
<tr>
<td>NTCP</td>
<td>CCGTCTTGCTGCCCCTTCT</td>
<td>GAACTCTGTTCCATGCTAGT</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>AGAGCTTGTCATGAGGGCAG</td>
<td>GGGGAGACATGAGAGAA</td>
</tr>
</tbody>
</table>

at 28 d (−53 and −54%, respectively, \(P<0.001\); Fig. 1(a)). No significant differences among the groups were observed for HDL-cholesterol concentrations (\(P>0.05\), data not shown). On the contrary, plasma concentrations of VLDL + LDL-cholesterol in rats fed the diets containing vegetable proteins, fibres or the combinations were markedly lower than those in the animals fed the control diet, at both 14 and 28 d of treatment, and the two combinations were more effective in lowering plasma cholesterol concentrations when compared with fibres alone (Fig. 1(b)).

No significant variations in TAG concentrations were detected, except for the group fed apple pectin, where a significant increase was observed at 28 d of dietary treatment (Fig. 1(c)).

Rats fed the diets containing oat fibres, apple pectin or the combinations had a lower cholesterol content in the liver compared with the control rats, whereas no variations were detected between the controls and the pea protein-fed animals (Fig. 2(a)). No changes in hepatic TAG content were instead observed among the groups (Fig. 2(b)).

**Effect of the diets on the hepatic mRNA concentration of genes involved in cholesterol metabolism**

In order to examine the possible mechanisms behind the different impacts of the vegetable-containing diets on cholesterolaemia, hepatic gene expression of several key enzymes and transporters involved in cholesterol and bile acid metabolism was evaluated (Table 3).

In the pectin group, hepatic mRNA concentrations of hydroxymethyl-glutaryl-CoA (HMG-CoA reductase) and LDL receptor were raised when compared with the control group (\(P<0.05\)). No differences among the groups were observed for mRNA levels of sterol regulatory element-binding protein-2...
The protein expression of hepatic CYP7A1 in the experimental groups is shown in Fig. 3. Rats fed the diets containing apple pectin and the two combinations (pea proteins + oat fibres and pea proteins + apple pectin) showed higher CYP7A1 protein expression compared with the controls ($P<0.05$). The group fed oat fibres displayed a trend towards higher expression when compared with the controls, but did not reach statistical significance.

Discussion

In the present study, each dietary treatment containing vegetable proteins and/or soluble fibres substantially lowered plasma cholesterol concentrations, if compared with the control diet containing animal proteins and insoluble fibres. The most effective cholesterol-lowering regimens were those combining pea proteins with oat fibres or apple pectin. Considering the hypocholesterolaemic effect of the dietary treatments containing the single effective ingredients (only pea proteins or only soluble fibres), it appears that the cholesterol reduction obtained with the combination of vegetable proteins and soluble fibres is likely to be due to an additive effect. Although this result can be considered somehow expected on the basis of previous studies testing the efficacy of the so-called portfolio diets (26–28), the possibility of a synergistic effect could not be excluded, based on the supposed mechanisms of action of these bioactive ingredients (25,29).

Indeed, the effect of these dietary components on cholesterol metabolism was explored in the present study, through quantitative real-time PCR.

Previous studies in rodents, performed by the present and other research groups, explored the effect of pea proteins on plasma lipids. Pea proteins generally displayed a marked hypocholesterolaemic activity (34,35), although this effect is not consistent in clinical studies (36,37). Altogether, these data suggest that the hypocholesterolaemic effect of the dietary soya protein isolate on cholesterol metabolism is likely to be due to an additive effect could not be excluded, based on the supposed mechanisms of action of these bioactive ingredients (25,29).

In the present study, we did not observe any significant transcriptional activation of SREBP-2 and its target genes, including the LDL receptor, in the group fed pea proteins, but we instead observed a strong activation of ABCG5/G8 (18,31). The present study confirmed the great efficacy of pea proteins in lowering plasma cholesterol concentrations, but failed in showing any effect on TAG. A possible explanation for the lack of hypotriacylglycerolaemic effect may reside in the very low basal TAG concentrations in rats, associated with the absence of a significant TAG rise in the control group that may have hindered a possible efficacy of pea proteins in modulating plasma TAG concentrations.

Legume proteins are believed to lower plasma cholesterol concentrations, at least in part, by LDL receptor activation (5).

Effect of the diets on hepatic cholesterol 7α-hydroxylase protein expression

The protein expression of hepatic CYP7A1 in the experimental groups is shown in Fig. 3. Rats fed the diets containing apple pectin and the two combinations (pea proteins + oat fibres and pea proteins + apple pectin) showed higher CYP7A1 protein expression compared with the controls ($P<0.05$). The group fed oat fibres displayed a trend towards higher expression when compared with the controls, but did not reach statistical significance.
believed to alter hepatic cholesterol homeostasis primarily by reducing dietary cholesterol uptake in the intestine and by increasing the faecal loss of bile acids, thus reducing the enterohpatic bile acid pool. As a consequence, cholesterol is rapidly catabolised in the hepatocyte to replenish the bile acid pool via the activation of CYP7A1, the rate-limiting enzyme in bile acid synthesis. Indeed, in the present study, a significant transcriptional increase of this enzyme was found in rats fed oat fibres and apple pectin. A significant reduction in hepatic cholesterol content was observed with both soluble fibres, and was significantly higher in rats fed pea proteins plus apple pectin feeding compared with the single ingredients, which was significant with respect to soluble fibres alone. The transcriptional activation of CYP7A1 observed in rats fed oat fibres or apple pectin was maintained in both treatments combining pea proteins and soluble fibres, and was significantly higher in rats fed pea proteins plus apple pectin than any other dietary regimen.

The combination of soluble fibres and pea proteins led to a further decrease of plasma cholesterol concentrations compared with the single ingredients, which was significant with respect to soluble fibres alone. The transcriptional activation of CYP7A1 observed in rats fed oat fibres or apple pectin was maintained in both treatments combining pea proteins and soluble fibres, and was significantly higher in rats fed pea proteins plus apple pectin than any other dietary regimen. To validate the transcriptional data obtained, Western blotting analysis of CYP7A1 expression was performed. The results were overall in line with the real-time PCR results, suggesting a good correlation between RNA and protein levels. The additional stimulation of CYP7A1 gene transcription by pea proteins plus apple pectin feeding may suggest that this dietary combination could have further increased the bile acid loss mediated by pectin alone, leading to raised bile acid synthesis. Interestingly, the increased transcriptional activation of ABCG5/G8 observed after feeding a pea protein-only diet was not maintained for the diets containing both soluble fibres and pea proteins. ABCG5/G8 expression is stimulated by liver X receptor in response to hepatic cholesterol overload to enhance biliary cholesterol secretion. The diets containing the combinations of pea proteins and soluble fibres significantly reduced the hepatic cholesterol content v. the treatment with pea proteins alone. This hepatic cholesterol reduction was found for oat fibre-fed rats, suggesting that the hypcholesterolaemic effect of oat fibres may be mostly consequent to reduced intestinal cholesterol absorption. Surprisingly, the dietary treatment with pectin significantly raised TAG plasma concentrations. Dietary treatments with pectin, and generally with soluble fibres, lower or do not modify TAG levels. However, it has been reported that soluble fibre feeding may cause the hepatic secretion of large, TAG-rich VLDL. We may hypothesise that this effect has also occurred in the present study, leading to a plasma TAG rise in pectin-fed rats. In this regard, it should also be noted that bile acid sequestrants, used in the clinic as hypcholesterolaemic drugs, often raised TAG concentrations by increasing VLDL production.

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Table 3. Relative mRNA concentrations of the hepatic genes involved in the lipid metabolism of rats fed, for 28 d, Nath’s hypercholesterolaemic diets, differing in protein and fibre sources.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Pea</th>
<th>Oat</th>
<th>Pectin</th>
<th>Pea + oat</th>
<th>Pea + pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG5</td>
<td>1·00</td>
<td>0·82</td>
<td>2·20</td>
<td>0·94</td>
<td>0·69</td>
<td>1·95</td>
</tr>
<tr>
<td>ABCG8</td>
<td>1·00</td>
<td>0·65</td>
<td>8·71</td>
<td>0·35</td>
<td>0·33</td>
<td>1·20</td>
</tr>
<tr>
<td>BSEP</td>
<td>1·00</td>
<td>0·26</td>
<td>0·86</td>
<td>0·05</td>
<td>0·17</td>
<td>0·90</td>
</tr>
<tr>
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<td>1·00</td>
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<td>1·47</td>
<td>0·05</td>
<td>0·05</td>
<td>2·00</td>
</tr>
<tr>
<td>HMG-CoA red</td>
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<td>0·42</td>
<td>0·17</td>
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<td>0·16</td>
<td>0·97</td>
<td>1·28</td>
</tr>
</tbody>
</table>

* Mean values were significantly different from those of the control diet (P<0.05).
† Mean value was significantly different from that of the control, oat, pectin and pea + pectin diets (P<0.05).
§ Values were normalised to the reference gene cyclophilin and are expressed relative to the levels in the control animals set as 1.

Fig. 3. Expression of cholesterol 7α-hydroxylase (CYP7A1) in the rat liver and representative image of CYP7A1 immunoblotting experiments. Microsomal membranes were prepared from liver homogenates and 100 μg of proteins were subjected to immunoblotting using a polyclonal antibody directed against CYP7A1. * Mean values were significantly different from those of the control diet (P<0.005). BiP, Ig heavy chain-binding protein.
may have caused the lack of ABCG5/G8 transcriptional activation induced by pea protein feeding.

Finally, no dietary regimens affected the expression of BSEP which mediates the canicular excretion of bile acids\(^{(42)}\). On the contrary, NTCP expression, the transporter that is primarily responsible for the hepatic uptake of bile acids\(^{(42)}\), was increased. NTCP expression is increased by agents stimulating the bile acid turnover, such as cholestyramine\(^{(43)}\), and it is reduced, similar to CYP7A1, by farnesoid X receptor, in the presence of high bile acid levels\(^{(44)}\). Increased cholesterol excretion or reduced bile acid absorption mediated by the dietary regimens could therefore be responsible for the observed effect.

In conclusion, pea proteins, apple pectin or oat fibres are dietary components extremely effective in reducing cholesterolaemia in rats. The combination of these ingredients in the diet results in an additional efficacy with respect to soluble fibres alone, mainly consequent to reduced cholesterol and bile acid absorption. The hypothesis that this type of combinations may be of clinical value is supported by recent data showing a moderate hypocholesterolaemic effect in subjects consuming low amounts of these combined ingredients\(^{(45)}\).

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References


